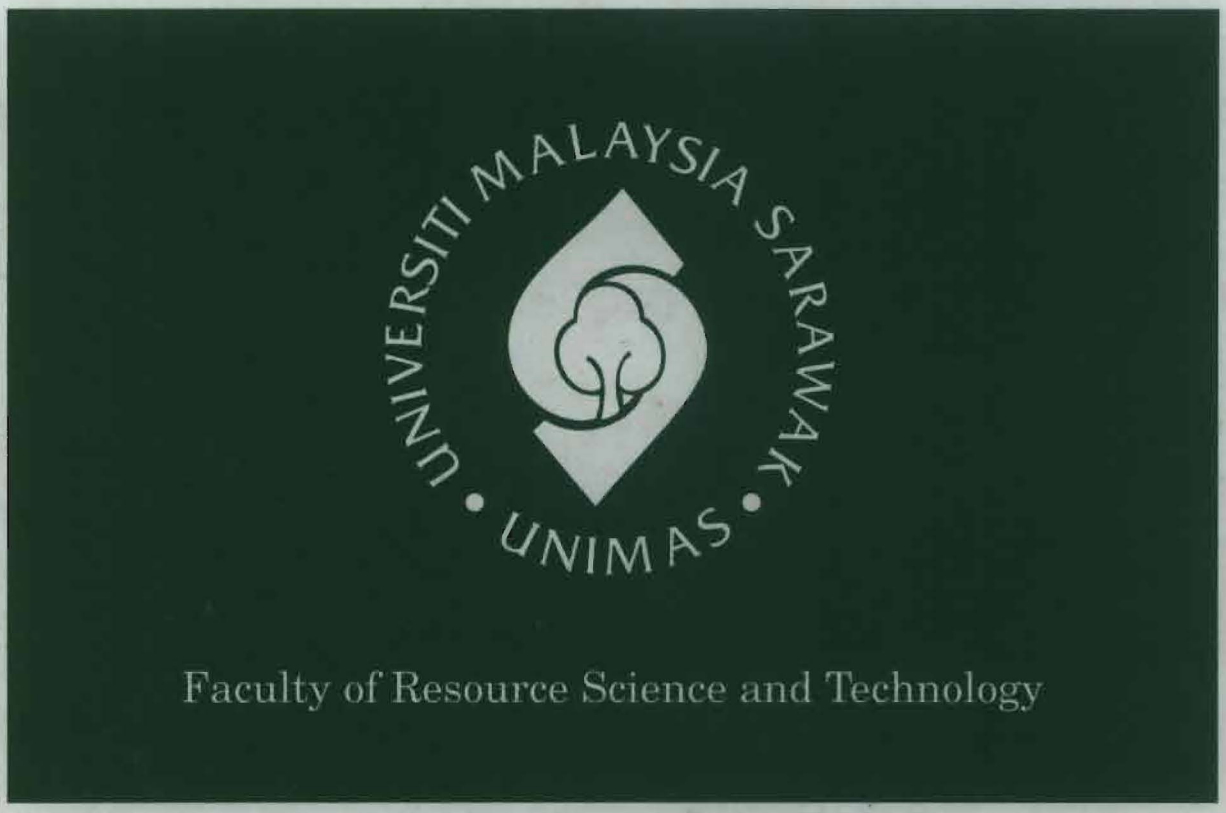

ISOLATION AND CHARACTERIZATION OF CELLULOSE DEGRADING BACTERIA FROM SAGO PITH WASTE

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µl	microliter
ml	milliliter
L	liter
mM	milimolar
M	molar
g	gram
bp	base pair
rpm	rotation per minute
C	Celsius
min	minute
mm	millimeter
mmol	milimol
U	unit
N	normality
V	volt

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ABSTRACT

Sago pith waste is one of the most abundant recyclable waste materials in our country. In this research, the most active cellulose degrading bacteria from sago pith waste was isolated and studied. Morphological characterization includes gram staining and some biochemical tests presumed the bacteria are pertained in genera *Staphylococcus*. For molecular characterization, genomic DNA was extracted from the bacteria and amplified by polymerase chain reaction before being sequenced. However, sequencing result failed to determine the specific species of the isolate. Crude cellulase enzymes was produced from the bacteria and assayed for its relative activity in different temperature, time and pH. Enzyme activities were expressed as units per milliliter (U/ml), where 1U was defined as 1 milimol of substrate oxidized per minute. The crude enzyme displayed highest activity in incubation at 30°C and is stable up to 60 to 80°C of temperature but with 50 to 80% decrease of activity. The optimal time for its activity is 8 minute while optimal pH is 7.0. Further research on the bacteria isolated and its cellulase enzymes produced should be conducted due to its potential in biotechnological applications and commercialization.

Keywords: sago pith waste, cellulose degrading bacteria, *Stahphylococcus*, cellulase.

ABSTRAK

Hampas sagu merupakan antara bahan sisa yang paling banyak didapati di negara kita. Dalam kajian ini, bakteria pendegradasi selulos yang paling aktif daripada hampas sagu diasingkan dan diselidik. Pengenalpastian secara morfologi termasuk 'gram staining' dan ujian biokimia menunjukkan bahawa bakteria diperolehi adalah dari genera Staphylococcus. Untuk pengenalpastian secara molekular, DNA genomik telah di ekstrak daripada bakteria tersebut dan digandakan melalui proses 'polymerase chain reaction (PCR)' sebelum dilakukan proses penjujukan. Walau bagaimanapun, hasil daripada penjujukan tersebut gagal menentukan dengan lebih spesifik spesies bakteria tersebut. Enzim selulase dihasilkan daripada bakteria tersebut dan dikaji aktivitinya di dalam suhu, masa dan pH yang berlainan. Aktiviti enzim dikira sebagai unit per mililiter (U/ml), di mana 1 U didefinisikan sebagai 1 milimol substrat yang dioksidakan per minit. Enzim menunjukkan kadar aktiviti paling tinggi pada suhu 30°C dan stabil sehingga suhu 60 ke 80°C tetapi dengan 50 hingga 80% penurunan aktiviti. Masa optima bagi aktiviti enzim ini adalah 8 minit manakala pH optima adalah 7.0. Kajian yang lebih lanjut berkaitan bakteria yang telah dipencilkan serta enzim selulase yang dihasilkan harus dilaksanakan memandangkan potensinya di dalam aplikasi bioteknologi dan pengkomersilan.

Kata kunci: hampas sagu, bakteria pendegradasi selulos, Staphylococcus, sellulase.

CHAPTER 1

Introduction

Sago is one of the main staple food crops in Southeast Asia and Pacific (Doelle, 1998). Sago industry had shown great contribution in developing our country economy. According to Bujang *et al.* (1996), sago starch industry becomes the main food industry which contributes more than 90% of country production. The biggest sago areas in Malaysia is Sarawak, which now is the world biggest exporter of sago, exporting about 25 000 to 45 000 tonnes of sago products annually. However, residues from its production tend to be discharge into the river or dumped at factory compound or site without any further uses (Vikineswary and Shim, 1994).

There are three types of waste that produced from sago bioprocessing industry, which are sago bark, sago waste water and sago pith residue or 'hampas'. Sago pith waste is obtained after extraction of starch from cellulosic cell walls of the trunk and it can be a very strong pollutant because of its cellulosic fibrous materials (Doelle, 1998). Therefore appropriate management or reutilization of this waste is very important in order to maintain environment health besides to gain benefits from converting the waste materials into more value added product.

One way to renew or make use of the waste from sago industry is by applying the concept of microbial degradation into this matter. Since microorganism plays a very important role in degrading the biomass such as cellulose in nature, the sago waste can be

properly controlled and benefited from when bacteria or fungi use and convert them into reducing sugar that can be subsequently used as substrate to produce valuable products such as enzymes, acids and alcohol. According to Coughlan, (1985) the possibility of using microorganism to enzymatically convert cellulose polymer into biofuels to offset energy shortages led to a widespread interest in cellulolytic microorganisms.

Microorganisms such as bacteria and fungi produce cellulase enzymes to hydrolyse cellulose components. In general, bacterial cellulases are constitutively produced, whereas fungal cellulases are produced only in the presence of cellulose. Isolation and characterization of cellulolytic microorganism had been undertaken for several decades to make sure the target to employ them for conversion of cellulosic waste can be achieved. Some of cellulolytic bacteria and fungi strains that have been isolated and characterized are like *Clostridium thermocellum* strain F1 (Ahsan *et al.,* 1996), *Acetobacter xylinum* strain KU1 (Oikawa *et al.,* 1997), *Trichoderma reesei* (Tomme *et al.,* 1988) and *Neocallimastic frontalis* (Pearce and Bauchop, 1985).

This project aims are to isolate the bacteria that can actively hydrolyzed cellulosic materials from sago pith waste as a source. The main objective is to identify and characterized the bacteria that can highly degrade cellulose from the sago pith waste samples. To achieve this, screening, isolation and characterization via morphological and molecular methods and also enzyme assay had been carried out.

CHAPTER 2

Literature review

2.1 *Metroxylon sagu* (sago palm)

Metroxylon sagu is the scientific name for sago palm plant. It is usually found in tropical regions especially in relatively humid low land areas. It is a perennial type of plant which grows well in swampy, acidic peat soils where few other crops can survive. Sago palm is a high producer of starch where it can produce in average of 160 kg of starch which can increase to 275 kg if cultivated in a well attended farm (Doelle, 1998). Sago palm is usually processed into raw sago starch, sago flour and other food products such as noodles and biscuit and even for animal feeds. In Malaysia, more than 90% of sago starch is produced in East Malaysia whereby Mukah in Sarawak is the largest producer with over 50% production of sago starch (Bujang *et al.*, 1996). Currently, sago exports ranks as the fourth biggest agricultural revenue earner for Sarawak, after oil palm, pepper, and cocoa. Sago brings in RM23.15 million in export earnings in 1993, overtaking the export of rubber in value terms in that particular year. There are two main species of sago palm in Sarawak, which are *M. rumphii* ('thorny') and *M. sagu* ('thornless') sagu. Sago palm is a very suitable candidate for reutilization or renewable resource as its waste residues usually have no commercial value and can be obtained easily from the processing area.

2.2 Sago pith waste

Sago pith waste or 'hampas' is a type of sago waste that can be obtained after extraction of starch from cellulosic walls of palm trunk. It is done after the cortex, rachis and leaflets had been removed from the sago trunk. It usually contains high amount of starch and cellulosic materials which amount 65.7 % of starch, 14.8 % of crude fibers and 1% of crude protein (Vikineswary and Shim, 1994). Sago 'hampas' usually found in bulk and dumped or sometimes used to feed the animals. It can be a very strong pollutant to the environment because of its high cellulosic materials (Doelle, 1998).

2.3 Cellulose

Cellulose is the most abundant organic macromolecules on earth. About 4×10^{10} tonnes of cellulose are produced annually mainly in higher plants making it 40% of total biomass produced each year by photosynthesis (Coughlan, 1984). Cellulose is found exclusively in plant cell walls in nature although it also can be produced by animals and bacteria. In a few cases it present in nearly pure state such as cotton bolls and mainly can be found embedded in a matrix of other structural biopolymers primarily hemicellulose and lignin.

Cellulose is composed of homopolymer of β-1,4-linked glucose residues in chair configuration.

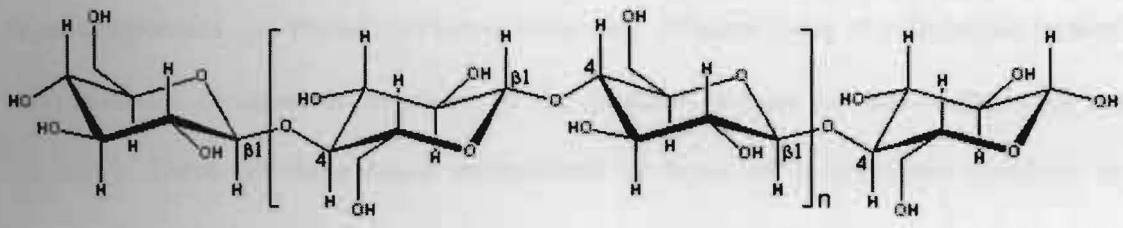

Figure 1: cellulose molecule structure

Each of glucose residues is rotated 180° with its neighbours along the main axis of the chain. The rotation of adjacent residue and β-1,4-linkage gives cellulose chain a ribbon-like structure stabilize by hydrogen bond. About 60 to 70 adjacent chains form crystalline microfibrils and bundles of microfibrils aggregate to form insoluble fibers, a character of cell walls of higher plants (Lee *et al.* 2002).

Cellulose is well known of its crystalline structure that describes the tight interaction of the cellulose chains because of the hydrogen and Van der Waals forces held together the structure. This make it packed sufficiently tightly to prevent penetration of other molecules such as water and enzymes. Naturally occurring cellulosic compounds are structurally heterogonous and have both amorphous and highly ordered crystalline regions. The degree of crystallinity varies with the source of the cellulose; the more crystalline regions are more resistant to enzymatic hydrolysis (Bok *et al.*, 1998). Hydrolysis of cellulose had been studied since past three decades for its economic potential in transformation of the renewable resource.

2.4 Cellulases

Cellulases are a group of hydrolytic enzymes capable of hydrolyzing cellulose to smaller sugar components. To degrade cellulose molecules, different types of cellulolytic system and cellulases components involved in the process because of the diversity of its structure. There are three major components or types of of cellulases involved in hydrolysis of native's cellulose which are endo-ß-glucanase (EC 3.2.1.4), exo-ß-glucanase (EC 3.2.1.91) and ß-glucosidase (EC 3.2.1.21).

Endo-β-1,4-glucanases or endoglucanases act by cutting at random at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends. These enzymes are glycoproteins with molecular weights ranging from 5,300-14,500. They are also referred to as carboxymethylcellulases (CMCase).

Exo-β-1,4-glucanases or exoglucanases acts in a processive manner on the reducing or nonreducing ends of cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products. Exoglucanases can also act on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure.

ß-Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose (Lee *et al.*, 2002). They are also known as cellobiases with molecular weights ranging from 50,000 to 41,000.

Cellulases are distinguished from other glycoside hydrolases by their ability to hydrolyze ß-1, 4-glucosidic bonds between glucosyl residues. The enzymatic breakage of the ß-1,4-glucosidic bonds in cellulose proceeds through an acid hydrolysis mechanism, using a proton donor and nucleophile or base (Birsan *et al.*, 1998). Cellullase enzymes have found variety of applications in production and processing industry such as textile industry, for biopolishing of fabrics and producing stonewash look of denims (Cavaco-Paolo, 1998). Besides, to de-inking the paper is also another emerging application of cellulases (Tolan and Foody, 1999).

2.5 Cellulose degrading bacteria

Microorganisms were the organism that responsible or exclusively carry the hydrolysis and utilization of celluloses in our nature. Microorganism that can degrade cellulose is abundant and ubiquitous in nature which includes fungi and bacteria. They produced cellulase enzymes to hydrolysed cellulose molecules. Not all microorganisms can produce significant quantities of cellulase to completely hydrolyzing crystalline cellulose. Only a few bacteria had been reported to produce significant cellulose activity, compared to fungi.

Various cellulolytic bacteria have been isolated from different sources such as plants, soils and ruminal animals. Those bacteria had been identified and characterized and many of them have shown high potential for application in industrial or environmental biotechnology. Some of the cellulolytic bacteria that have been isolated are for example; *Bacillus Amyloliquefaciens* UMAS 1002 (Apun *et al.,* 2000), which can produce extracellular enzymes that can hydrolyse CMC and break down sago pith waste, *Clostridium thermocellum* strain F1 (Ahsan *et al.*, 1996), which able to degrade crystalline cellulose and *Acetobacter xylinum strain* KU1 (Oikawa *et al.*, 1997), which can produced a high level of extracellular endo-β-glucanase.

CHAPTER 3

Materials and Methods

3.1 Samples

Samples of sago pith waste that had been used in this study were obtained from one of sago processing factory in Mukah, Sarawak. The samples were in a wet form and stored under the temperature of 4°C. A serial dilution with sterile distilled water (1:9 ml) was carried out before doing isolation of bacteria from the samples to minimize the numbers of microorganisms inside the sample.

3.2 Culture and screening

In order to culture the bacteria, 200 µl of the diluted sample was spreaded onto a selective media agar containing carboxymethyl cellulose (CMC). This media was prepared by mixing 0.2% (w/v) of yeast extract, 0.1% (w/v) KH_2PO_4, 0.5% (w/v) $MgSO_4$, and 0.5% (w/v) of CMC with distilled water (Apun *et al.,* 2000). The plate was incubated in 37°C for 24 hours. To screen for the highest degraders of cellulose, the culture plates was flooded with Congo red solution for 15 minutes and then washed with 1M sodium chloride (Teather and Wood, 1982). The colony that produced the biggest halo was isolated and transferred to a new media for further analysis. The colony was continuously subcultured to obtain pure culture of bacteria.

3.3 Morphology characterization

3.3.1 Gram staining

Gram staining, a test to determine whether the bacteria is gram negative or gram positive bacteria was firstly conducted in morphological characterization in this research. Gram staining was carried out by preparing the bacteria smears on a glass slide that was fixed by gently heating it on a flame. The slide was then flooded with crystal violet for 30 second, before washing it with distilled water. After that, grams iodine was applied for 30 second and washed again with distilled water. Then a drop of acetone was applied to decolouring the smear and immediately rinsing it with distilled water. Final step is counterstained the slide with safranin for about 1minute and then washed with distilled water. The slide was air dried before being viewed under light microscope.

3.3.2 Biochemical test

i. **Catalase test**

This test is to determine the presence of enzyme catalase and/ or peroxidase. One pure colony of bacteria was placed on a clean, glass slide. A drop of 30% H_2O_2 applied on the colony and positive result is showed by production of immediate bubbling on the colony.

ii. Oxidase test

This test was conducted using oxidase reagent as suggested by the manufacturer (DAFICO Laboratories). The reading of the color changes was taken within 30 seconds to ensure accuracy.

iii. Triple sugar iron(TSI) test

TSI slant agar which was prepared by adding 5.94 g TSI agar powder into 100 ml dH_20 was inoculated with one colony of the bacteria by stabbing the butt and streaking the slant. Then the test tube was incubated for 24 hours at 37°C.

iv. Citrate utilization test

Simmons citrate agar was prepared and one colony of the bacteria was streaked on the agar. The agar plate then incubated at 37°C for 48 hours.

v. Glucose-phenol red test

Phenol red broth was prepared by dissolving 1.5 g phenol red powder into 100 ml dH_2O and placed in test tubes. Then 0.1 g of D-glucose was added to each tube. Durham tube was added to each tube to examine the presence of gas produced. One colony of bacteria inoculated in the broth and incubated for 24 hours at 37°C.

vi. Mannitol-phenol red test

Phenol red broth was prepared similar with glucose test except replacing 0.1 g of D-glucose by 0.1 g of mannitol powder added into each tube. One colony of bacteria inoculated in the broth and incubated for 24 hours at 37°C.

vii. Methyl red-Vogues-Proskauer (MR-VP) test

Methyl red-Vogues Proskauer (MR-VP) broth was prepared by dissolving 0.15 g methyl red powders into 10ml distilled water. The broth was placed in universal bottle, 10 ml each and autoclaved at 121°C for 30 minutes. One colony of the microorganism was inoculated into the broth by flamed loop inoculation. Then the bottles were incubated for 48 hours at 37°C. One ml of the broth culture then transferred into a clean test tube for VP test by adding Barrit's solution A and B and the colour changes being observed. The remaining of the culture was used for MR test by addition of MR indicator.

3.4 Molecular identification

3.4.1 Total genomic DNA extraction

In order to extract genomic DNA from the bacteria, the method based on Ausubel *et al.* (1992), with some modification was used in this study. Firstly, 1.5 ml of the overnight culture was centrifuged at 13 000 rpm for 1 minute. After that the cell pellet was resuspended with 567 µl of TE buffer (pH 8.0) by repeat pipetting. Then 5 µl of Proteinase K and 10 µl of 25% SDS added into the suspension and gently mixed and incubated at 60°C for 20 minutes. 50 µl of lysozyme was added along into the mixture. Then 500 µl PCI (phenol/chloroform/Isoamylalcohol) mixtures were added and mixed by gentle inversion. After that the solution was centrifuged at 13,000 rpm for 1 minute. Then 200 µl of the most upper part from the 3 layers then pipetted out into a fresh sterile 1.5 ml microcentrifuge tube. About 200 µl of 5 M Na-Ac (pH 4.5-5.5) added together with 400 µl of isopropanol. The solution then gently mixed and left at room temperature for 5 minutes. The solution was centrifuged again at 13,000 rpm for 7 minutes and the supernatant discarded. . The DNA pellet obtained washed with 70% cold ethanol before centrifuging them at 13,000 rpm for 5 minutes. The supernatant was discarded and the pellet allowed drying at room temperature. The DNA pellet then dissolved with 50 µl TE buffer. The extracted total genomic DNA then visualized using 1% (w/v) agarose gel electrophoresis.

3.4.2 Polymerase chain reaction (PCR) amplification

In this research, PCR amplification was performed using DNA extracted previously from the bacteria isolate as a template. According to Weisburg *et al.* (1991), the use of rDNA sequences from PCR is a powerful tool to deduce phylogenetic and evolutionary relationships among bacteria. Therefore, for molecular identification of the isolate, 16sRNA primers were used in the PCR amplification process. The primers which were 25 pmol/µl of PA forward primer (5'-AGA-GTT-TGA-TCC-TGG-CTC-AG- 3') and 25 pmol/µl of PH reverse primer (5'-AAG-GAG-GTG-ATC-CAG-CCG-CA-3') which was stated as a pair of primer that capable to amplify a wide variety of bacterial taxa (Weisburg *et al.*, 1991) was used in this research.

PCR reaction mixture that had been used consist of 2.5 µl of 10X PCR buffer (Fermentas), 1.5 µl of 25 mM MgCl2 (Fermentas), 0.5 µl of 10 mM dNTPs (Fermentas), 1.25 µl of 25 pmol/µl of Primer PA, 1.25 µl of 25 pmol/µl of Primer PH, 16µl of Sterile distilled water, 1µl of Taq Polymerase (Fermentas) and 1µl of template DNA. The final volume is 25 µl and the amplification run for 30 cycles.

Table 1 : Primer sets for molecular identification

Primers (25 pmol/µl)	sequences
PA forward primer	(5'-AGA-GTT-TGA-TCC-TGG-CTC-AG- 3')
PH reverse primer	(5'-AAG-GAG-GTG-ATC-CAG-CCG-CA-3'

The summary of the PCR reaction mixture was shown in the table below:

Table 2 : PCR reaction mixture contents

PCR Reagent	Quantity Per Reaction
10 X PCR buffer (Fermentas)	2.5µl
25 mM MgCl2 (Fermentas)	1.5µl
10 mM dNTPs(Fermentas)	0.5µl
25 pmol/µl of Primer PA	1.25 µl
25 pmol/µl of Primer PH	1.25 µl
Sterile distilled water	16µl
Taq Polymerase (Fermentas)	1µl
Template DNA	1µl
Total Final Volume	25µl

3.4.3 DNA sequencing

The PCR product obtained was firstly purified using DNA purification kit (Fermentas) before sent for direct automated sequencing at First BASE Laboratories Sdn.Bhd. for sequencing the bacterial DNA. The sequence result was then analysed using BLAST to find sequence similarity with pre-existing sequences of bacteria from NCBI GenBank.